BACTERIOPHAGE-RESISTANT MUTANTS OF PASTEURELLA PESTIS AND THEIR PROPERTIES

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BACTERIOPHAGE-RESISTANT MUTANTS OF PAST. PESTIS AND THEIR PROPERTIES

[Following is the translation of an article by A. I. Volosivetz, "Mikrob" All-Union Scientific-Research Institute, Saratov (Director - Prof. N. I. Nikolayev), published in the Russian language periodical Byull. Eksptl. Biol. i Med. (Bulletin of Experimental Biology and Medicine), No 11, 1963, pp.107-10. It was received by the editors on 12 Nov. 1962 and was presented by N. N. Zhukov-Verezhnikov, USSR Academy of Medical Sciences. Translation performed by Sp/6 Charles T. Ostertag Jr.]

In a previous report the results were presented of the study of the biological properties of three plague phages. In accordance with the results obtained the lytic activity of these phages (EV, 1-17, D'Herelle) is found in correlation with the adsorption capability and duration of intracellular development. All these phages make up one serological group. Adsorption of the main mass of phage particles takes place in 5-8 minutes (EV and 1-17) and 8-10 minutes (D'Herelle). Clarification of the properties of these phages made it possible to use them in experiments on the isolation and characterization of phage-resistant mutants.

The present article sheds light on the establishment of the frequency of spontaneous mutations of plague bacteria from phage-sensitivity to phage-resistance, and also on the study of the properties of phage-resistant mutants in connection with the properties of the phages.

Experimental Methods

The possibility of the mutation genesis of phage-resistant variants of Past. pestis is made apparent with the help of the "replication" technique [4]. With this aim, 18-24 hour colonies of plague causative agent (100 strains) were transferred with the help of a "stamp" to agar dishes in which bacteriophage was preliminarily deposited. The resistant colonies which appeared on the dish-replica were repeatedly sown by loop onto agar plates with phage and then were reseeded several times on agar plates without phage.

The frequency of spontaneous mutations from phage-sensitivity to phage-

resistance was determined by the Luria and Delbruck method with Newcombe's modification. For this, 24 hour broth cultres of plague causative agent were placed on agar plates with phage already applied. Control seedings of the same volume were carried out on agar plates without phage. The calculation of resistant colonies was performed after 48 hours since after 24 hours (as the author recommends) it wasn't possible to detect any signs of growth. The phage-resistant mutants which emerged, as in the first method, were repeatedly passed through plates with phage, and then the colonies which grew the second time were emulsified in broth and were reseeded several times on agar plates without phage. The frequency of formation of phage-resistant mutants was calculated according to the formula: $a = -(\ln P_o)/N$, where a is the mutation frequency in one division cycle of the bacterial cell, P_o is the number of cultures without mutants, and N is the average number of colonies by the end of growth in broth prior to reseeding on dishes with phage.

The properties of the initial strains were studied by the generally accepted method of investigating the plague causative agent [1, 2].

Results of Experiments

Resistant mutants of bacteria appeared in the amount of 3-4 colonies among both the cellular population of virulent as well as avirulent strains of the plague microbe. The frequency of formation of phage-resistant mutants, calculated according to the cited formula, comprised:

$$a_{EV} = -(\ln 2) \cdot (\ln P_o)/N = -(\ln 2) \cdot (\ln 86)/500 = 6 \cdot 10^{-8}$$
in the other case $2 \cdot 10^{-8}$

$$a_{1\text{-}17} = -(\ln 2) \cdot (\ln P_o)/N = -(\ln 2)\ (\ln 80)/500 = 6 \cdot 10^{-8}$$
in the other case $2 \cdot 10^{-8}$

$$a_{\text{D'Herelle}} = -(\ln \cdot 2)\ (\ln \cdot P_o)/N = -(\ln \cdot 2)(\ln \cdot [83])/500 = 6 \cdot 10^{-8}$$
in the other case $2 \cdot 10^{-8}$.

It is apparent from this that the number of colony-mutants makes up some fraction of a percentage or units of percentages to the number of reseeded cells and the frequency of formation of phage-resistant mutants of P. pestis is insignificant.

The following phase of the work was a study of the properties of some resistant mutants. The mutants were denoted by the generally accepted method [3]. We observed three variants of colonies which differed in their morphological features on agar.

1. Light-yellow with a raised hilly center and a considerably expressed flat or scalloped edge -- the R-form.

2. Light-yellow fine-grained, or with a smooth granularity, colonies with a raised center and in the majority of cases a coil-shaped edge which is rarely even -- the OR-form.

3. Completely flat colorless colonies with a raised center and an uniform edge -- the S- form. Growth on broth fluctuated depending on the morphology of the colonies on agar. In colonies of the first and second type, agglutinated growth was observed and a precipitate on the bottom which, following shaking, rose in the form of flakes. As a rule colonies of the third type produced a cloudiness in the broth. A study of the morphology of the bacteria showed a considerable polymorphism of gram negative bacilli -- from clearly expressed ovoids to coccoid bacilli. Active motility of the bacteria, observed in a hanging drop, was noted in four out of the 13 tested mutants (418/1-17$_{(1)}$, 460/EV, 499/1-17 $_{(1)}$, 1303/D), while in the remaining ones Brownian movement or non motility was recorded. Lytic ability of the mutants was checked by the application of non diluted phages of EV, 1-17, and D'Herelle and the pseudotuberculosis phage on a lawn of the culture (see table).

Thus the majority of mutants are resistant to all the phages and even to the pseudotuberculosis phage. The 1309/EV mutant is resistant only to the EV phage but is lyzed by the remaining two. The second mutant 1378/1-17 is partially lyzed by all the phages. The resistance of the majority of mutants to the phages studied testifies to the antigenic closeness of the phages. In the mutants obtained the ability to adsorb phage particles was shown. It is known that several resistant variants are capable of adsorbing that phage to which they are resistant [3]. In our experiment mutant 1378/1-17 turned out to be capable of adsorbing the phage particles of all the phages. Following the seeding of the remaining variants by the method of agar layers, sterile patches didn't emerge.

Keeping in mind the data by many authors that the plague microbe under the influence of one or another factor, in particular phage, often becomes altered with a tendency toward the pseudotuberculosis causative agent, we studied the biochemical activity of the mutants and their ability to grow on differential-diagnostic media (acid-deficient agar, peptone-free agar, Otten medium, Mayeda medium No 2, Kolya-Bel'kura medium, litmus milk, urea).

Analyzing the data from table 2, the phage-resistant mutants can be divided into two groups based on their biochemical properties and the ability to grow on differential-diagnostic media. The first includes three media: 418/1-17 $_{(1)}$, 499/1-17$_{(1)}$, and 1303/D. All the remaining ones belong to the second group.

The mutants of the first group, which at the moment of isolation have a hilly light-yellow surface and a sharply expressed scalloped edge, fermented rhamnose in 24 hours, gave a blue pigmentation to Otten's medium with the addition of litmus tincture, grew on Mayeda's No 2 medium and acid-deficient agar, decomposed urea in 1-4 days, reduced nitrates to nitrites, and almost completely reduced methylene blue. On peptone-free agar the mutants of the first group grew in the eighth dilution, while the remaining ones grew in the second dilution and variant BP-5Zh/D in the fourth dilution.

Mutants of both groups decomposed the Kolya-Bel'kura medium.

Attention is warranted to the relationship of the mutants of the second group to rhamnose and glycerin. The majority of them fermented rhamnose, but in later periods (7th to 10th day) in comparison with mutants of the first group. Three mutants, 499/1-17 (2), 460/1-17, and 460/EV, lost the ability to decompose glycerol while the initial cultures fermented this alcohol in three days. Mutant 1107 decomposed glycerol only on the 16th day. From what has been stated it follows that mutants of the first group, based on their biochemical activity and ability to grow on differential-diagnostic media, displayed properties which are inherent to the causative agent of pseudotuberculosis. The study of the serological properties of the obtained mutants in the classic reaction with agglutinating sera, and also of the immune sera to the mutants with the initial strains and mutants showed that both the initial strains and the mutants gave a positive agglutination reaction in the same dilutions.

The concluding phase of the study of the properties of the isolated mutants was checking their pathogenicity for rabbits and guinea pigs. The death of the rabbits was caused by mutants of the first group in two days following the intravenous introduction of a 0.1 ml dose of a one billion two day suspension in a physiological solution. Mutants of the second group didn't cause the death of rabbits. Guinea pigs died with the introduction of mutant 1378/1-17 in a dose of 10,000 and 1,000,000,000 microbial bodies while the other mutants didn't cause death in these animals. A repeated administration of 200 Dcl of a virulent strain to these animals caused their death in 4-14 days. This testifies to the lack of immunogenic properties in the mutants obtained.

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[The following English summary appeared with the Russian article.]

An inquiry was made into spontaneous mutation from the bacteriophage-sensitivity to bacteriophage-resistance in Past. pestis of different virulence. According to preliminary data, mutants resistant to bacteriophage appeared irrespective of the degree of the initial strain virulence and of the bacteriophage species used. Variants were isolated from the mutants which by their chemical properties and capacity to grow on differential-diagnostic media and high pathogenicity for rabbits could be referred to pseudotuberculosis causative agents.

Table 1

Sensitivity of Phage-Resistant Mutants to Bacteriophages

Mutants	Phage			
	EV	1-17	D'Erellya	Pseudo-tubercu-losis
499/1-17 (1)	-	-	-	+
1309/EV	-	±	±	-
179/EV	-	-	-	-
1107/1-17	-	-	-	-
418/1-17 (1)	-	-	-	+
495/1-17	-	-	-	-
1303/D	-	-	-	+
1378/1-17	±	±	±	±
460/EV	-	-	-	-
BP-BZh/D	-	-	-	-
499/1-17 (2)	-	-	-	±
418/1-17 (2)	-	-	-	±
460/1-17	-	-	-	-

Table 2

Properties of Phage Resistant Mutants

Table 2

Properties of Phage Resistant Mutants

Strain	Glucose	Maltose	Mannitol	Lactose	Saccharose	Glycerol	Rhamnose	Nitrification	Denitrification	Reduction of blue	Kolya-Bel'kura Medium	Otten Medium	Mayeda Medium No 2	Acid-hungry Agar	Peptoneless Agar	Urea
499/1-17(1)	+++	++++	++	-	-	-	+3d	+	-	nrs	++++	Blue	eg	wg	8	+3d
1309/EV	++	++++	++	-	-	+3d	-	++++	++++	-	++++	Red	-	-	2	-
179/EV	++	++	++	-	-	+3d	-	-	-	-	++++	"	-	-	2	-
1107/1-17	++	+++	++	-	-	+16d	+7d	++++	++++	-	++++	"	-	-	2	-
418/1-17(1)	++	+++	++	-	-	+3d	+1d	+	-	nrs	++++	Blue	eg	wg	8	+1d
495/1-17	++	+++	++	-	-	+2d	+10d	-	-	nrs	++++	Red	-	-	2	-
1303/D	++	+++	++	-	-	+3d	+1d	+	-	nrs	++++	Blue	eg	wg	8	+4d
1378/1-17	++	+++	++	-	-	+3d	-	-	-	-	++++	Red	-	-	2	-
460/EV	+	+	++	-	-	-	+7d	-	-	-	++++	"	-	-	2	-
460/1-17	-	++	++	-	-	-	+7d	-	-	-	++++	"	-	-	2	-
BP-5Zh/D	++	+	++	-	-	+3d	+10d	-	-	-	++++	"	-	-	4	-
499/1-17(2)	++	+	++	-	-	-	-	-	-	-	++++	"	-	-	2	-
418/1-17(2)	++	+	++	-	-	+10d	-	+	+	-	++++	"	-	-	2	-

Legend: - negative reaction; + partial decomposition; ++ weak decomposition; +++ complete decomposition (intensive staining); ++++ sharply expressed decomposition; numbers with letter "d" denote on which day decomposition took place; nrs incomplete reduction of methylene blue; eg expressed growth; wg weak growth.